MECHANISTIC INVESTIGATION ON THERAGNOSTIC APPLICATION OF COMBINATION OF DRUGS-LOADED HOLLOW MESOPOROUS SILICA NANOPARTICLES IN BREAST CANCER CELL LINE

A SYNOPSIS

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T.M. VISWANATHAN
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(DEEMED TO BE UNIVERSITY)

ANAND NAGAR, KRISHNANKOIL – 626 126

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1. INTRODUCTION

Cancer persists as a major health obstacle in worldwide and has led to the demise of millions of individuals in the twenty-first century. It is marked by the unrestrained proliferation and splitting of irregular cells, which may give rise to growths and intrude on neighboring tissues or organs [1].

Breast cancer is the most prevalent type of cancer among women worldwide, and it is also the most common cause of death in woman population [2]. It accounts for up to 36% of cancer cases in women globally and in 2018, around 2.089 million women were diagnosed with it globally [3]. According to data from Globocan for 2020, breast cancer accounted for 13.5% (17.8 lakh) of all cancer cases and 10.6% (90,408) of all cancer-related deaths in India [4].

Anti-cancer drugs used in breast cancer treatment have a major drawback of causing severe side effects, such as fatigue, nausea, diarrhea, neutropenia, elevated liver enzymes, conjunctival disorders, and epistaxis. To enhance breast cancer therapy, a multifaceted strategy is necessary, which involves better comprehension of the biology of breast cancer and its development and progression mechanisms, identifying novel targets, and devising innovative treatment methods.

Nutraceuticals refer to natural substances that possess biological activity and are utilized for preventing or treating diseases with minimal side effects [5]. Curcumin, a polyphenolic compound present in Curcuma longa's rhizome, is broken down in the liver and gut into curcumin glucuronide, curcumin sulfate, and hexahydrocurcuminol. Curcumin has anti-cancer properties and has demonstrated effectiveness against various cancer types such as breast, pancreatic, colorectal, prostate, and lung cancer, as well as multiple myeloma and head and neck squamous cell carcinoma [6]. Besides, curcumin has therapeutic benefits against Alzheimer's disease, inflammation, atherosclerosis, diabetes, infection-related ailments, and chronic bacterial prostatitis [7].

Metals and metal complexes play a significant role in our environment, with some being essential for physiological functions, while the benefits of others remain unclear. However, some metals have been identified as toxic. Scientific research has revealed that prolonged exposure to certain metallic compounds can result in various

types of cancer, including breast cancer [8]. There is an increasing body of evidence that external factors such as exposure to carcinogens, including metal compounds, contribute to the development of breast cancer [9].

Gallium (Ga) is a metal compound that is considered a high potential candidate for anticancer therapy, second only to platinum [10–12] Gallium compounds bind with transferrin and are taken up by cells through the transferrin receptor to form transferrin-gallium (Tf-Ga) complexes [13,14]. This complex interferes with the normal uptake of the transferrin-iron complex, leading to iron deprivation in cancer cells [15]. Gallium-based metallodrugs, including gallium nitrate, gallium chloride, gallium maltolate, tris (8-quinolonato) gallium (III), or KP46, have been used as anticancer agents, with gallium maltolate and KP46 being evaluated in preclinical trials [16,17] and gallium III nitrate going up to clinical trial phase II [18]. While gallium nitrate shows promise as a potential treatment for breast cancer, further research is required to determine its optimal use and potential side effects.

Guanidine is a molecular transporter that binds to inositol dimers of the plasma membrane, which enhances cell penetration and delivers various compounds to the cells [19]. This compound interacts with adenine and thiamin phosphate residues in the DNA helix, damaging DNA and leading to cell death [20]. Guanidine is being studied for its potential use in treating breast cancer, as it has been shown to have anticancer effects in preclinical studies. Specifically, it inhibits the activity of ribonucleotide reductase, an enzyme necessary for DNA synthesis and cell proliferation. Preclinical studies have demonstrated that guanidine carbonate can reduce the viability of breast cancer cells in vitro and inhibit breast tumor growth in animal models.

Abnormal blood vessels in the tumor microenvironment can accelerate tumor growth and hinder the delivery and effectiveness of therapeutic drugs due to limited drug availability caused by the absence of lymph vessels and reduced blood vessels. The presence of stromal cells and hypoxic regions can further impair blood flow, reducing drug exposure to the tumor site [21]. One strategy to overcome this problem is to use nanocarriers to deliver therapeutic molecules in a targeted manner with sustained release.

Hollow mesoporous silica nanoparticles (HMSNP) are a highly versatile and desirable platform for drug delivery systems due to their variety, accessibility, and biocompatibility. HMSNP is non-toxic and has a large surface area, adjustable pore size, excellent physicochemical stability, and chemically modifiable surfaces [22,23]. Silica nanoparticles can be coated with various polymers such as polyethylene glycol (PEG), polycaprolactone (PCL), dextran, chitosan, polyethyleneimine (PEI), and 3-aminopropyl triethoxysilane (APTES) to improve drug loading, retention time, release, and prevent aggregation. Another approach is to attach drug molecules to the pore or surface of HMSNP through stimulus-responsive linkages [24,25]. HMSNP is an efficient nanocarrier that enables greater storage and release of chemical substances and chemotherapeutic drugs [26–28].

Our research is to understand the combination effect of gallium-curcumin and guanidine-curcumin complex loaded hollow mesoporous silica nanoparticles against breast cancer. We aim to recognize the important role of combinatorial therapy in breast cancer and overcome the barriers that limit the clinical success of this vital anticancer treatment.

2. REVIEW OF LITERATURE

2.1 Breast cancer and current treatment

Breast cancer is a type of cancer that forms in the clumps of the breast. It is one of the most common cancers among women, but it can also occur in men. The treatment of breast cancer depends on various factors such as the stage of the cancer, the type of breast cancer, and the overall health of the patient. Current treatment options for breast cancer may include surgery, radiation therapy, chemotherapy, targeted therapy, and hormone therapy. Surgery is often the first line of treatment and involves the removal of the tumor and nearby lymph nodes. Radiation therapy uses high-energy beams to kill cancer cells, while chemotherapy uses drugs to destroy cancer cells throughout the body. Targeted therapy focuses on specific characteristics of cancer cells to block their growth and spread, and hormone therapy works by blocking the effects of hormones on breast cancer cells. Regular screening and early detection are crucial in improving the chances of successful treatment and outcomes for breast cancer patients, Łukasiewicz et al. [29].

2.2 New strategies development for breast cancer

The development of breast cancer treatments is a multifaceted process that encompasses extensive research, including the study of the underlying biology of the disease to identify potential treatment targets. Targeted therapies, such as HER2 inhibitors, have emerged as effective options for specific breast cancer subtypes, leading to improved outcomes. Immunotherapy has revolutionized treatment by leveraging the body's immune system to combat cancer cells, Pusztai et al. [30]. Combination therapies, which involve using multiple treatment modalities in a coordinated manner, are being explored to enhance efficacy. Clinical trials play a crucial role in testing and validating new approaches, while collaboration among healthcare professionals fosters the exchange of knowledge and drives progress in the field of breast cancer treatment, Ward et al. [31].

2.3 Curcumin in breast cancer treatment

Curcumin, a compound found in turmeric, has demonstrated the ability to induce cell death in breast cancer cells. It activates apoptosis, a programmed cell death mechanism, and interferes with the cell cycle progression of cancer cells. Additionally, curcumin has been found to possess anti-inflammatory and antioxidant properties, which can help reduce chronic inflammation and oxidative stress, both of which are associated with cancer development and progression. By modulating these pathways, curcumin may help inhibit the growth and survival of breast cancer cells, Liu and Chen [32], Harini et al. [33].

2.4 Gallium - Modern therapeutics for Breast cancer

Gallium nitrate has emerged as a promising compound for breast cancer treatment due to its ability to induce cell death through multiple mechanisms. By inhibiting ribonucleotide reductase, gallium nitrate disrupts DNA synthesis and impairs cancer cell proliferation. Its interference with calcium metabolism triggers cellular stress and activates apoptotic pathways, leading to cancer cell death. Additionally, gallium nitrate hinders angiogenesis, depriving cancer cells of essential nutrients. While further research is necessary to fully comprehend its efficacy and safety, these diverse mechanisms highlight gallium nitrate's potential as a therapeutic option for breast cancer, Qi et al. [34].

2.5 Guanidine - Future Medicine for Breast cancer

Guanidine, an investigational compound, holds promise in inducing cell death in breast cancer cells. It has been primarily studied as a radio sensitizer, potentially amplifying the efficacy of radiation therapy by increasing the sensitivity of cancer cells to radiation. The specific mechanisms by which guanidine induces cell death in breast cancer are still not fully understood and require further investigation. Guanidine mechanisms include disruption of DNA repair processes, modulation of cell survival signaling pathways, and increased generation of reactive oxygen species. However, more research, including preclinical and clinical trials, is needed to unravel the precise mechanisms and potential therapeutic applications of guanidine in breast cancer treatment, Gomes et al. [35].

2.6 Nano drug delivery system

Drug delivery systems are integral to current breast cancer treatment approaches. They are designed to efficiently transport anti-cancer drugs to the tumor site, enhancing their effectiveness while reducing side effects. Nanoparticles, targeted delivery, drug-eluting implants, combination therapies, hydrogels, and injectable depots are among the various strategies utilized. Ongoing advancements in drug delivery systems continue to enhance the efficacy and safety of breast cancer treatments, Kingsley et al. [36].

2.7 Hollow Mesoporous Silica nanoparticles

Hollow mesoporous silica nanoparticles (HMSN) have garnered interest in the field of breast cancer research due to their unique properties and potential applications. HMSN possess a hollow interior and a porous structure, allowing for high drug-loading capacity, large surface area and controlled drug release on breast cancer cells. This targeted approach increases the accumulation of therapeutic agents at the tumor site while minimizing off-target effects. HMSN can also be loaded with various anti-cancer drugs, including chemotherapy drugs or gene therapies, enabling their efficient delivery and enhancing treatment efficacy. Ongoing research is exploring the potential of HMSN in improving the effectiveness of breast cancer treatment and reducing side effects, highlighting their promising role in the field of nanomedicine, Li et al. [37].

2.8 Combination of drugs in breast cancer treatment

Combination drug therapies play a vital role in breast cancer treatment, allowing for enhanced efficacy and targeting multiple aspects of the disease. Nowadays, different combinations of chemotherapy, hormonal therapy, targeted therapy, and immunotherapy are common for breast cancers. These combinations maximize treatment outcomes, overcome drug resistance, and provide comprehensive blockade against cancer cells. The selection of combination therapies depends on factors such as tumor subtype, stage of the disease, and patient characteristics. Ongoing research focuses on refining combination regimens to improve outcomes and minimize side effects, advancing the field of breast cancer treatment, Fisusi and Akala [38].

2.9 Apoptosis and protein interaction in breast cancer cell death

Apoptosis, a regulated process of cell death, plays a pivotal role in eliminating abnormal or damaged cells, including breast cancer cells. Protein interactions within breast cancer cells contribute to the intricate regulation of apoptosis and ultimately determine the fate of these cells, Ouyang et al. [39]. The Bcl-2 family of proteins, which includes both pro-apoptotic and anti-apoptotic members, plays a crucial role in this process. Imbalances in the interactions between these proteins can disrupt the apoptotic signaling pathways, leading to uncontrolled cell growth and survival. Moreover, protein interactions involving caspases, the key mediators of apoptosis, are also critical for executing cell death, Thomadaki and Scorilas [40]. Consequently, targeting the protein interactions involved in apoptosis has emerged as a promising strategy for breast cancer treatment. Ongoing research in this field aims to unravel the complex protein networks underlying breast cancer cell death and identify novel therapeutic targets to enhance treatment outcomes.

3. OBJECTIVES

1. Synthesis and characterization of combination of drugs-loaded HMSNAP to analyze drug loading and drug release kinetics.

2. Combination of Gallium and curcumin-loaded HMSNAP (GaC-HMSNAP) and Guanidine and curcumin-loaded HMSNAP (GuC-HMSNAP) treatment on MCF-7 cells and assessment of their toxicity.

3. Evaluation of the effect of drugs-loaded nanoparticles on the alteration of endoplasmic reticulum, mitochondrial associated proteins and phosphorylation of oncoproteins in MCF-7 cells.

4. Validation of drugs-loaded HMSNAP induced apoptotic proteins in MCF-7 cells through western blotting.

4. RESEARCH GAP IDENTIFICATION

The rising prevalence of breast cancer has necessitated the search for more effective medications with fewer adverse effects. Extensive research has been conducted in this area to explore the potential of natural, metal, and inorganic drugs, aiming to uncover their biological activities. However, thus far, none of these studies have successfully established a comprehensive understanding of their drug complexes or the mechanisms by which they eliminate breast cancer cells through the use of nanocarriers. As a groundbreaking approach, we have ventured into the uncharted territory of utilizing hollow mesoporous silica nanoparticles loaded with a combination of drugs to induce cell death specifically in breast cancer cells. This innovative strategy holds tremendous promise in the ongoing battle against breast cancer, offering a potential breakthrough in the development of more targeted and efficient treatments.

5. ORGANIZATION OF THE THESIS

This thesis consists of five chapters, which are arranged in the following structure

Chapter 1

The chapter 1 explains the introduction, which covers breast cancer as a prevalent and deadly cancer affecting women, accounting for around 25% of all cancers diagnosed in women. Current treatments include surgery, chemotherapy, radiation therapy, and hormone therapy, while new strategies such as immunotherapy and targeted therapy are being developed. Promising approaches for treatment include the use of natural compounds, gallium compounds, guanidine, and nanocarriers for drug delivery. Dysregulation of protein interaction and apoptosis mechanisms can

lead to abnormal cell growth, immune evasion, and resistance to treatment, making it crucial to understand these mechanisms for developing novel therapies.

Chapter 2

Chapter 2 explains the literature review, which focuses on the topics of cancer, breast cancer, and their statistics. We then investigate the role of natural compounds, gallium, and guanidine are inducing breast cancer cell death. Additionally, we discuss the use of nanocarriers in drug delivery systems, particularly hollow mesoporous silica nanoparticles. Finally, we explain the mechanisms of cell death, including mitochondrial and endoplasmic reticulum-mediated apoptosis, as well as the role of protein expression in breast cancer cell death.

Chapter 3

The chapter 3 analyzed the synthesis and characterization of gallium-curcumin complex and guanidine-curcumin complex loaded hollow mesoporous silica nanoparticles. The synthesis of hollow mesoporous silica nanoparticles (HMSNP) was attained using the sol-gel emulsion methods. The prepared hollow mesoporous silica nanoparticles with loaded drugs before and after functionalisation were characterised by scanning electron microscopy (SEM), high resolution transmission electron microscopy (HRTEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), Dynamic Light Scattering (DLS), and Zeta Potential. Then, the surface elemental analysis of the drug-loaded nanocarrier was analysed by X-ray photoelectron spectrum. Finally, the specific surface areas from Brunauer–Emmett–Teller (SBET) and Barrett–Joyner–Halenda (BJH) analysis were used to determine the surface area, the pore-size distribution, and the pore volume. The drug complex loaded with HMSNAP was assessed through ^{1}H nuclear magnetic resonance (NMR).

Chapter 4

Chapter 4 of the study analyzes the in vitro performance of the gallium-curcumin complex and guanidine-curcumin complex loaded hollow mesoporous silica nanoparticles. The chapter covers drug loading, drug release, cytotoxicity assay, and apoptosis assay. To investigate drug loading, the drug-loaded HMSNAP was kept in ethanol for up to 48 hours, and the amount of drug complex loading was calculated using a spectrophotometer. For drug release studies, the drug complex-loaded

HMSNAP was dispersed in phosphate-buffered saline (PBS) at three different pH levels: 3, 6, and 7.4. The percentage of drug release was calculated every 6 hours for up to 66 hours. *In vitro* cytotoxicity and apoptosis assays were analyzed using MTT and Acridine orange/Ethidium bromide assays. These studies used to evaluate the toxicity of lead molecules such as gallium-curcumin, guanidine-curcumin, and complex-loaded nanoparticles. Finally, these *in vitro* evaluation results are used to protein validation studies. (Figure 1(a,b) and 2(a,b))

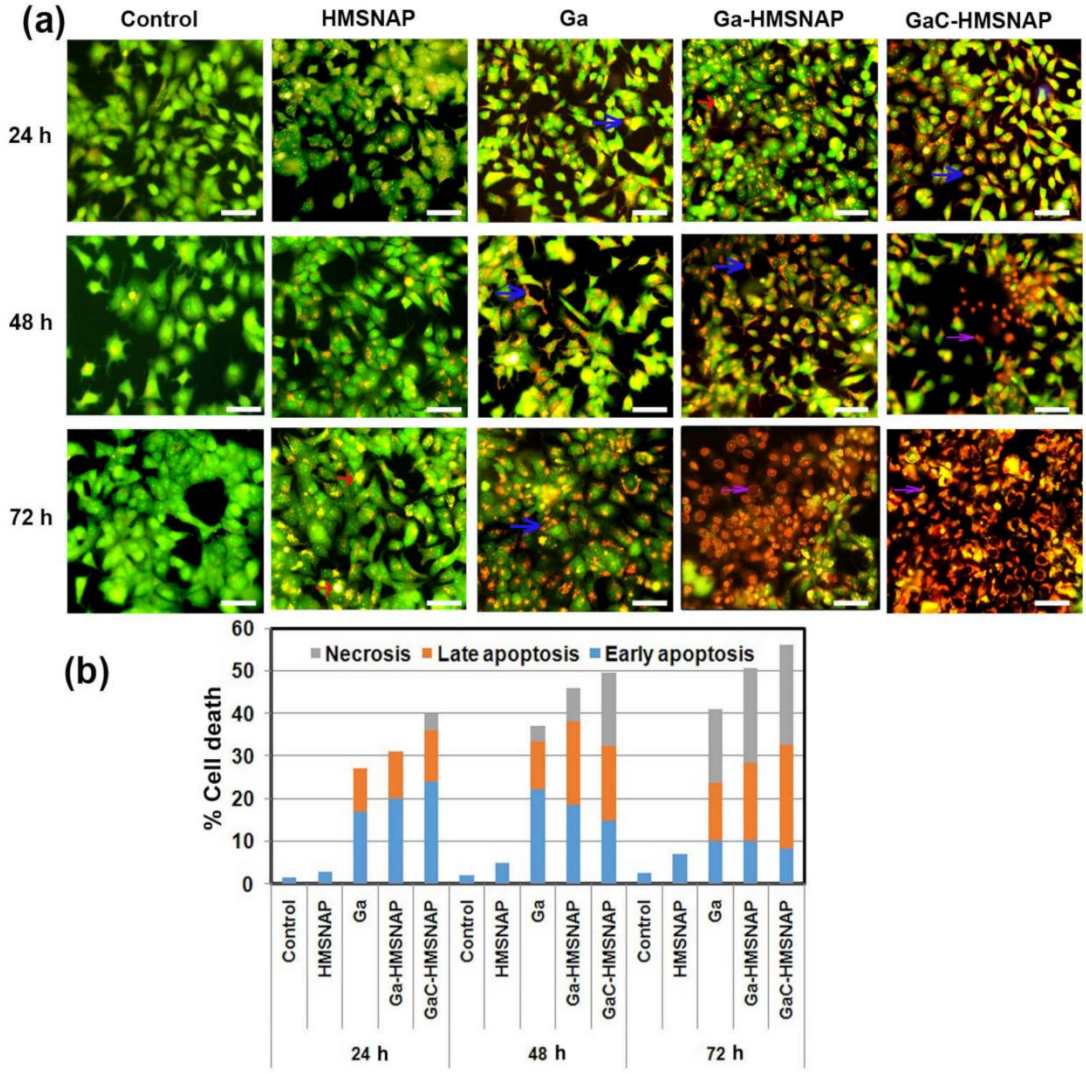

Figure 1. (a) Photomicrographs of acridine orange displaying the HMSNAP and drug-loaded HMSNAP-induced cell death in MCF-7 cells at different time intervals. The arrow marks the blue colour, representing early apoptosis, the orange, which represents late apoptosis, and grey, representing necrosis of MCF-7 cells (b) The graph represents the percentage of early and late apoptosis and necrosis in HMSNAP and drug-loaded HMSNAP-induced cell death in MCF-7 cells at different time intervals.

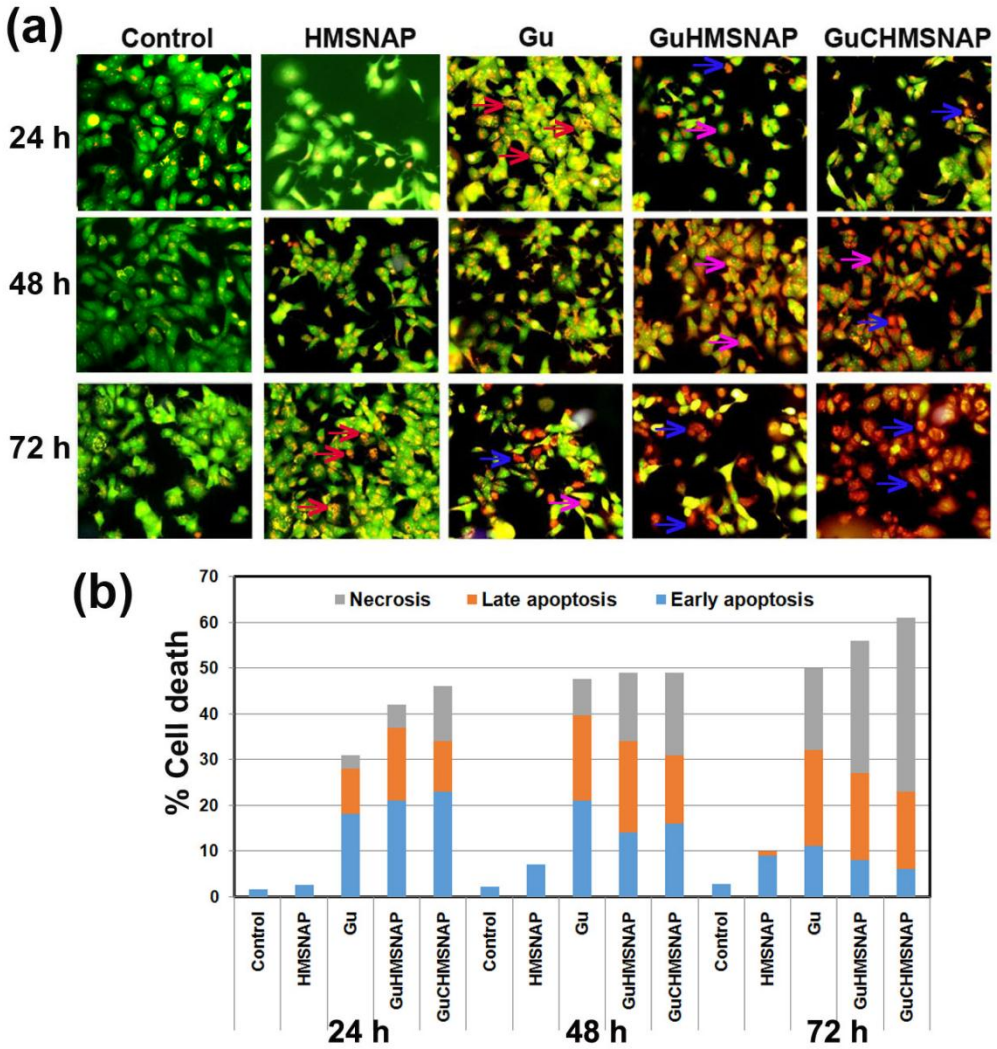

Figure 2. Photomicrographs of acridine orange displaying HMSNAP and drug-loaded HMSNAP induced cell death in MCF-7 cells at different time intervals. The arrow marked with a blue colour represents early apoptosis, the orange colour represents late apoptosis, and the gray colour represents necrosis of MCF-7 cells. (a) The graph represents the percentage of early and late apoptosis and necrosis in HMSNAP and drug-loaded HMSNAP-induced cell death in MCF-7 cells at different time intervals (b).

Chapter 5

Chapter 5 of the study investigated that the protein validation performance of the gallium-curcumin complex and guanidine-curcumin complex loaded hollow mesoporous silica nanoparticles. The MCF-7 cells treated with drug-loaded HMSNAP (GaC-HMSNAP and GuC-HMSNAP)-persuaded proteins were analysed through western blotting. The results suggest that drug complexes altered protein expression in Ser471 of Akt, Ser259 of c-Raf, and Ser241 of PDK1, phosphorylation in GSK-3

Ser9, cleaved caspases, and cleaved PARP, which then partially induces intrinsic cell death in MCF-7. As a whole, our results demonstrate that combination of drugs loaded is an efficient nanocarrier for effectively inducing cancer cell death. (Figure 3(a-e), 4(a,b) and 5(a,b))

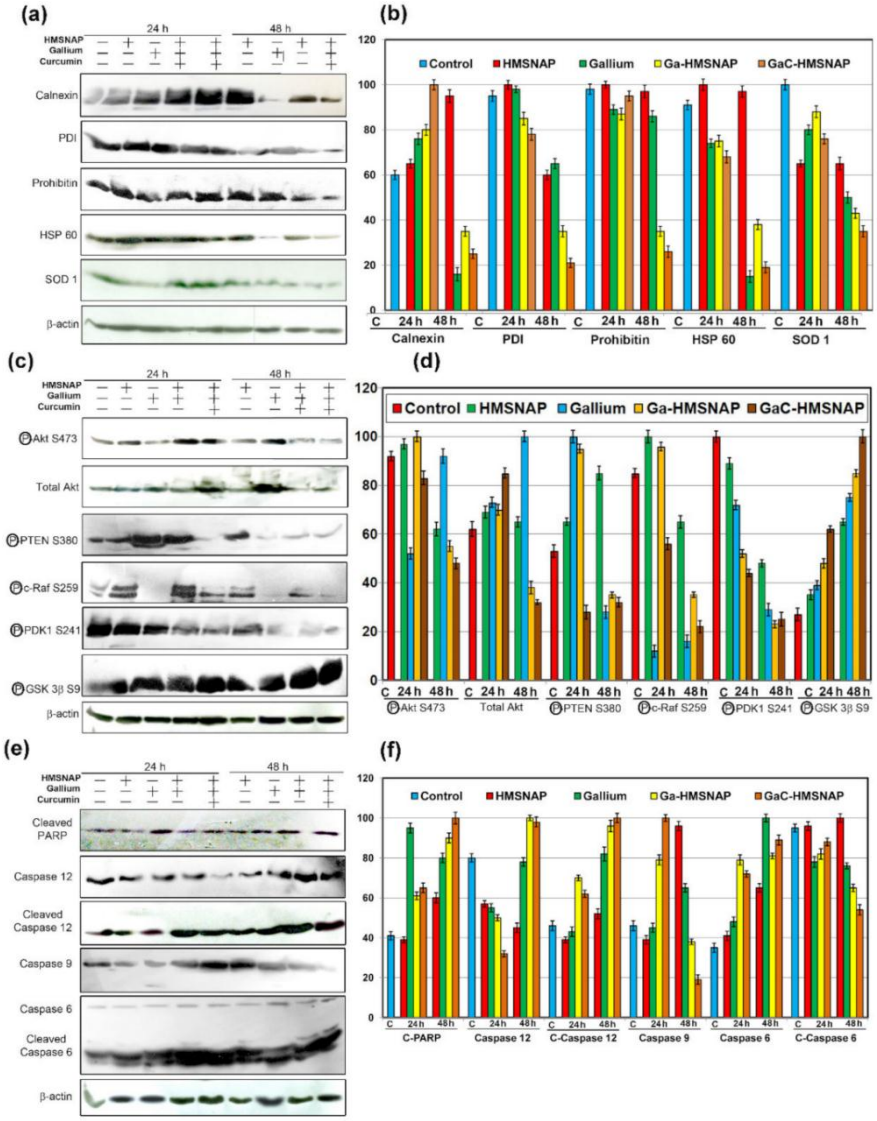

Figure 3. Western blot analysis used to determine the (a,b) phosphorylation of oncoproteins and tumour-suppressor proteins, (c,d) ER and mitochondrial proteins, and (e,f) apoptotic proteins in MCF-7 cells treated with HMSNAP and drug-loaded HMSNAP at 12 h and 24 h intervals, respectively. The graph represents the densitometric analysis of each protein compared with the respective control and calculates the percentage manipulation.

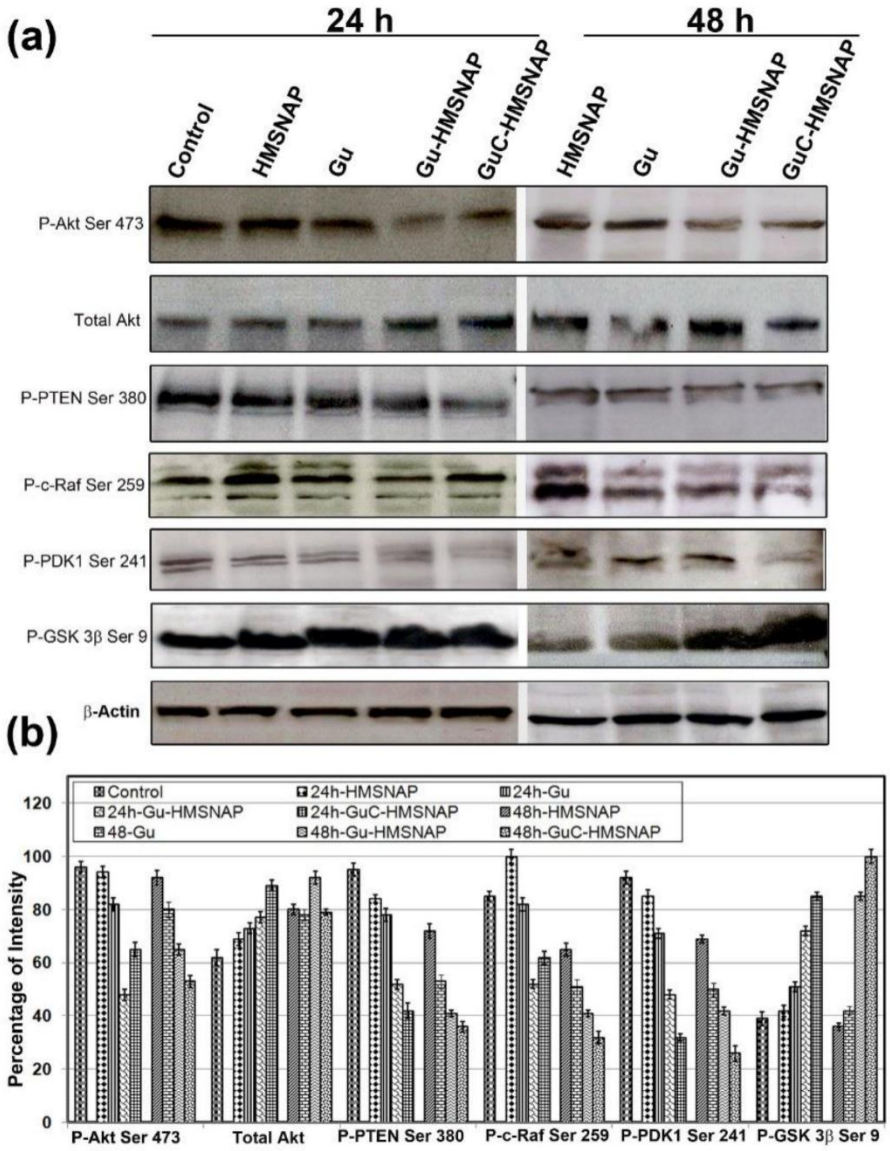

Figure 4. Western blot analysis was used to determine the phosphorylation of oncoproteins and tumour-suppressing proteins in MCF-7 cells treated with HMSNAP and drug-loaded HMSNAP at24 h and 48 h incubation. (a) The graph represents the densitometric analysis of each protein as compared with the respective control and calculates the percentage manipulation (b).

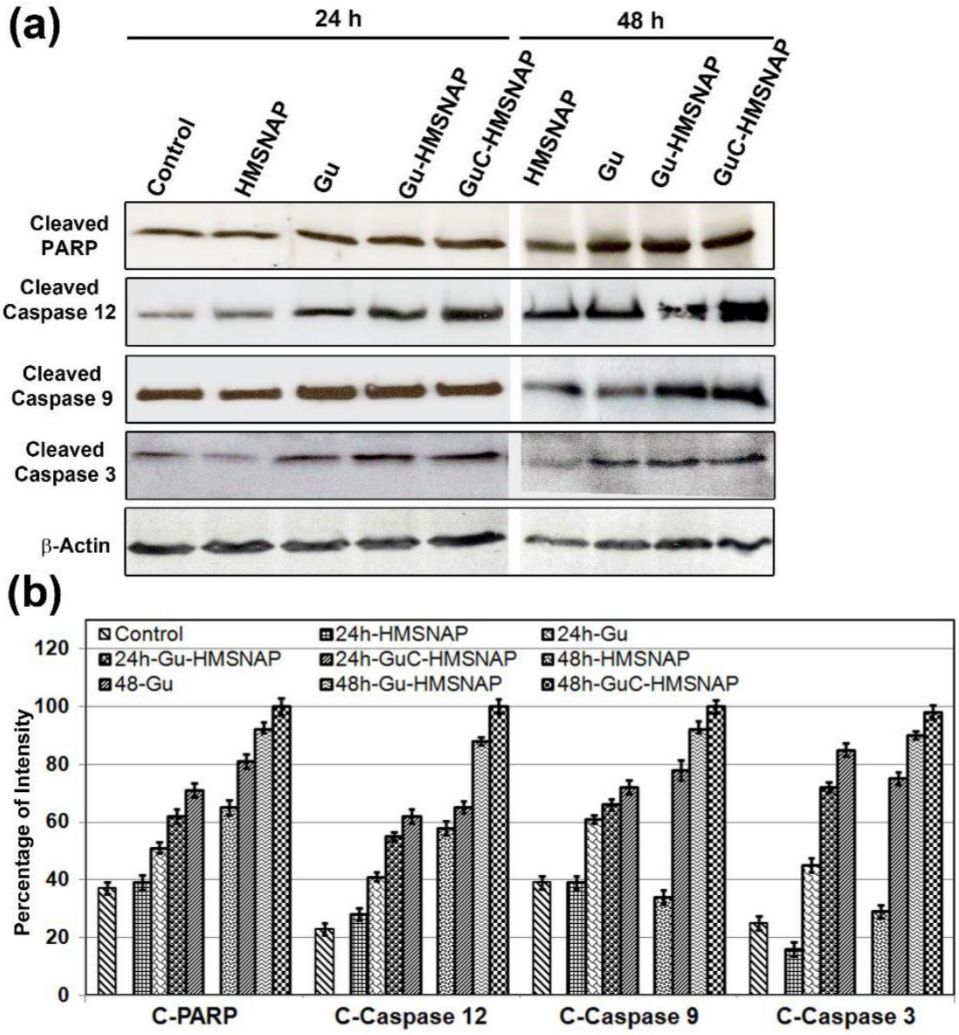

Figure 5. Western blot analysis of apoptotic proteins in MCF-7 cells treated with HMSNAP and drug-loaded HMSNAP at 24 h and 48 h intervals (a). The graph represents the densitometric analysis of each protein compared with the respective control and calculates the percentage manipulation (b).

Chapter 6

The chapter 6 explore the conclusion of the present work which highlights the co-delivery efficacy of a combination of drugs loaded with HMSNAP, aiming to induce cell death in breast cancer cells. We conducted characterization studies to assess various parameters such as mesosphere size, nanopores, surface charges, surface area, pore volume, and drug complex analysis.

These results confirmed modifications in these characteristics, indicating successful drug loading onto HMSNAP. To evaluate the effectiveness of the drug-

loaded HMSNAP, we performed in vitro cytotoxicity and apoptosis assays, which demonstrated significant cell death induced by the drugs. Additionally, western blot analysis revealed alterations in proteins associated with cell death pathways, further supporting the effectiveness of the drug-loaded HMSNAP as a potential nanocarrier for breast cancer therapy. These findings highlight the promising role of this drug delivery system in improving treatment outcomes for breast cancer patients.

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List of Publication

1. **T.M. Viswanathan**, V. Krishnakumar, D. Senthilkumar, K. Chitradevi, R. Vijayabhaskar, V.R. Kannan, N.S. Kumar, K. Sundar, S. Kunjiappan, E. Babkiewicz, P. Maszczyk, and T. Kathiresan, Combinatorial Delivery of Gallium (III) Nitrate and Curcumin Complex-Loaded Hollow Mesoporous Silica Nanoparticles for Breast Cancer Treatment, *Nanomaterials*, **12** (9) (2022), 1–23.

2. **T.M. Viswanathan**, K. Chitradevi, A. Zochedh, R. Vijayabhaskar, S. Sukumaran, S. Kunjiappan, N.S. Kumar, K. Sundar, E. Babkiewicz, P. Maszczyk, and T. Kathiresan, Guanidine–Curcumin Complex-Loaded Amine-Functionalised Hollow Mesoporous Silica Nanoparticles for Breast Cancer Therapy, *Cancers*, **14** (14) (2022),.

3. **T.M. Viswanathan**, A. Zochedh, K. Chandran, S. Sukumaran, A.B. Sultan, and T. Kathiresan, Investigation of Guanidine-Curcumin Complex Based on Quantum Chemicals, Pharmacokinetics and Molecular Docking Simulation against Breast Cancer: A Theoretical Approach, *Asian Journal of Chemistry*, **35** (5) (2023), 1189–1198.